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Eijlander, Robyn T.; Abee, Tjakko; Kuipers, Oscar P.

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Bacterial spores in food: how phenotypic variability complicates prediction of spore properties and bacterial behavior

Robyn T Eijlander^{1,2}, Tjakko Abbe^{1,3} and Oscar P Kuipers^{1,2}

Bacillus spores are a known cause of food spoilage and their increased resistance poses a major challenge in efficient elimination. Recent studies on bacterial cultures at the single cell level have revealed how minor differences in essential spore properties, such as core water content or germinant receptor levels, can cause the observed differences in spore germination and outgrowth behavior. Moreover, heterogeneous behavior is influenced by commonly accepted food preservation techniques, such as heating or the usage of weak organic acids. Understanding the underlying molecular mechanisms and key players involved in phenotypic heterogeneity of spores, while taking the spore's history into account, will improve predictability of the spore's behavior to various treatments and triggers.

Addresses

¹ Top Institute Food and Nutrition (TIFN), Nieuwe Kanaal 9A, 6709 PA Wageningen, The Netherlands

² Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

³ Laboratory of Food Microbiology, Wageningen University and Research Centre, Bomenweg 2, 6703 HD Wageningen, The Netherlands

Corresponding author: Kuipers, Oscar P (O.P.Kuipers@rug.nl)

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Introduction

In the harsh and everchanging environment of for instance the soil or the animal gut, bacteria of the *Bacillus* genus can adopt several strategies to ensure their individual survival and, even more importantly, the survival of the population. Complex cascades involving precise levels of (regulator) proteins, metabolites, expression of specific gene sets, and signaling molecules allow for the adaptation of a bacterium to its specific environment. They can achieve this by employing various strategies, including the take up of external DNA [1], chemotaxis [2,3], motility, biofilm formation [4] or the formation of dormant, highly resistant entities called spores [5]. Which of these strategies is adopted by the cell, is dependent on a multitude of factors

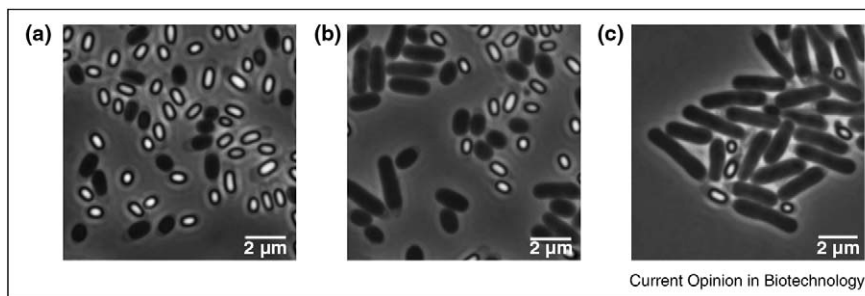
in which stochastic fluctuations in the genetic network as well as regulatory feedback mechanisms play essential roles [6,7]. Otherwise referred to as phenotypic heterogeneity (reviewed in [8]), this variation in adaptation within the same bacterial population increases the chances of survival of the species at question.

In this review, the importance of phenotypic variation in *Bacillus* spores is discussed, with focus on recent advances in gaining new insight in the molecular mechanisms for understanding the process of heterogeneity and factors involved. The important consequences of such phenotypic bacterial variation for the food industry have recently been pointed out [9]. It is through spores that foodborne pathogenic *Bacillus* species can survive stress conditions during preservation treatments such as heating, pressure or acidification. Phenotypic heterogeneity in spore properties hinders the predictability of spore behavior to such treatments (Figure 1). Insufficient inactivation and subsequent germination of these spores upon exposure to nutrients results in outgrowth and multiplication of these bacteria, with great risk of food spoilage and food poisoning after consumption [10]. *Clostridium* spores are another well known cause of food spoilage, but will not be discussed extensively in this review. This is mainly because the bulk of data regarding heterogeneity in spore properties and germination behavior concerns *Bacilli*. For recent reports on *Clostridium* spore (germination) characteristics we refer to Ramirez and Abel-Santos [11] and Paredes-Sabja *et al.* [12].

Heterogeneous response of *Bacilli* spores to (industrially relevant) treatments

Control of spores is one of the main problems in food preservation and complete inactivation is often impossible without affecting food quality and structure. Spores can survive a wide range of treatments including wet and dry heat, high hydrostatic pressure, desiccation, UV and γ -radiation, and antimicrobial compounds that rapidly kill vegetative cells. Moreover, spore properties and germination efficiency are clearly affected by differences in environmental conditions, as has been described for temperature variations during sporulation [13,14] as well as for the presence or absence of certain nutrients or chemical compounds in the sporulation medium [15,16] or the pH of the medium [17,18]. A deeper understanding of the mechanisms involved in spore resistance, adaptation and killing (and heterogeneity there-in) may lead to improved models for spore behavior prediction. In particular, the identification of specific factors involved,

Figure 1



Phase contrast pictures of *Bacillus cereus* cells and spores, indicating three levels of heterogeneity in germination. (a) Heterogeneity on the level of germination initiation, which is visualized by the transition from phase-bright to phase-dark spores. (b) Heterogeneity on the level of outgrowth. (c) Heterogeneity in germination and outgrowth, clearly showing dormant, phase bright spores among fully germinated and grown spores into vegetative cells. The scale bar in the bottom right of each picture indicates 2 µm.

the conditions and regulation of their expression as well as their roles in adaptation and resistance may aid in the assessment of specific spore properties and their behavior and response to treatments.

Dessicate to withstand heat

Resistance of spores towards a variety of stresses is made possible by specific spore core and cortex properties and several different mechanisms [19,20]. For example, one of the main contributors to wet heat resistance is the low water content in the spore core [21,22]. For long it has been suggested that the dehydrated state of the spore core results in a glass-like state [23,24] that would enable stabilization of proteins against thermal denaturation. Several attempts to detect and prove the glass-like state were unsuccessful [25,26] until a recent report showed that the spore core water mobility level is too high for such a state to occur [27^{••}]. Quantification of the hydration level in the spore core furthermore indicated that the suggested protein stabilization cannot take place [27^{••}] and rather implies that the core dehydration acts indirectly through the immobilization of proteins. Differences in core water content between individual spores of the same population consequently results in phenotypic heterogeneity in wet heat resistance [28[•]]. This is further reflected by a broad range of lag times before fast and complete release of the spores depot of dipicolinic acid (DPA) after wet heat treatment [29,30], which is an essential step in the spore's germination process. The mechanism of spore killing by wet heat is unknown, although recent experimental evidence points towards damage to one or more key proteins involved in metabolism [30], as minor changes in the structure of spore proteins were observed before DPA release [31[•]], while abrupt and major protein denaturation takes place only after DPA release, when the spores are already dead [29,30,32]. This suggests, however, that differences in the spore's water content do not play a role in the observed wet heat resistance heterogeneity, unless the

low water content is somehow involved in the protection of this/these key protein(s) and that minor changes in core water content affect this level of protection. Thus, key targets of wet heat inactivation remain to be elucidated. And also, a careful assessment of a number of controlled and uncontrolled factors (such as thermal history of the spores, adaptation or recovery medium pH and/or composition) should be taken into consideration [33].

Spore germination heterogeneity

Treatments as discussed above are not necessarily designed to directly kill spores, but rather to have them commit to the process of germination. This will abolish a large part of the spore's resistance and facilitate killing of the unwanted germs. In nature, dormant spores can commit to the irreversible process of germination in response to the presence of nutrients in the form of amino acids, sugars or purine nucleosides, indicating that conditions have improved for further proliferation. The mechanism of this commitment, the cascade of signal transduction and the intrinsic control mechanisms are not yet fully known. However, many factors involved have been identified and our understanding of control mechanisms that prevent premature germination is increasing. For instance, the alanine racemase, Alr, of *Bacillus anthracis* and *Bacillus thuringiensis* was found to suppress premature germination in the presence of low levels of the germinant L-alanine by converting this into the germination inhibitor D-alanine [34,35]. This indicates that germination commitment is only triggered when a threshold level of available nutrients is reached. These nutrients are bound by receptor complexes that are present in the inner membrane of the spore. Typically consisting of three proteins that are each essential for receptor activity [36,37], these Ger complexes exist in low numbers (~25 molecules per spore [38]) and display various nutrient specificities. Furthermore, a role in High Hydrostatic Pressure-induced germination has been described, with the notion that this germination pathway

can be by-passed at high pressures, for example, higher than 500 MPa [39]. The variety in Ger receptor complexes in *Bacilli* and *Clostridia* species as well as their interaction and activity and several germination strategies have recently been reviewed by Ross and Abel-Santos [40[•]], and thus we shall not discuss this in great detail. Our focus, however, lies on the role these receptor complexes play in the observed heterogeneity in spore germination.

Role of Ger receptor complexes in germination heterogeneity

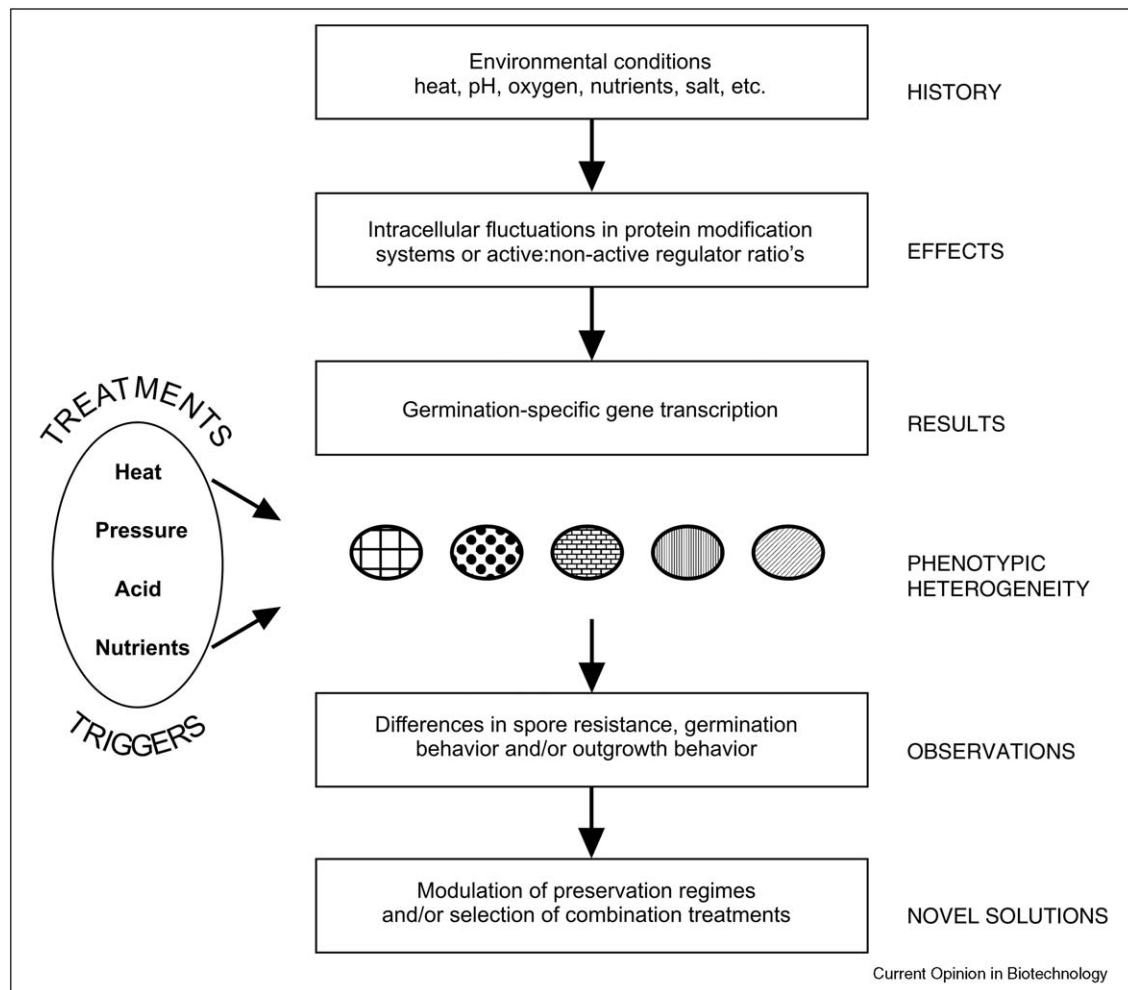
One of the first measurable events of spore germination after exposure to nutrients, is the spore's rapid release of DPA. Between individual spores large variations in time before this release takes place have been reported and attributed to, among other reasons, differences in germinant receptor protein levels [41]. Previous studies in *B. subtilis* already demonstrated that increasing Ger protein levels can result in faster germination responses [42]. However, this response is highly dependent on i) the concentration and type of nutrient germinants added, ii) the specific Ger complex at study, and iii) the level of overproduction, as too high levels of Ger proteins can in fact negatively influence germination [42]. Furthermore, it has been proposed that interaction between proteins of heterologous receptor complexes can result in the amplification of the signal that induces the germination response [43,44]. Taken together, this emphasizes the importance of a crucial balance between the number of Ger receptor proteins, the types of Ger receptor complexes and the level of available nutrients for optimal germination efficiency. Furthermore, positioning or conformational changes of the receptor proteins might play an additional important role [45]. In nature, stochastic fluctuations in the number of Ger receptor complexes or their components, caused by epigenetic variations between individual spores, can result in differences in spore behavior. It is safe to assume that these fluctuations are influenced by the history of the spores, as was shown for the expression of specific *ger* operons in *B. cereus* [16] and that this can affect the efficiency of germination (discussed in [46[•]]) (Figure 2). The underlying molecular mechanism for these stochastic fluctuations in germination-specific gene expression remains to be elucidated, although it seems plausible that stimulatory feedback loops during the sporulation process play an important role. The influence of sporulation history should be included in these studies to obtain a better understanding of factors influencing spore heterogeneity for spore behavior prediction models and specific attention should also be given to sporulation and germination behavior in (model) foods [47], since this aspect has been largely neglected up to now. The approach described in Figure 2 may eventually lead to modulation and/or development of new (combination) preservation regimes, more than likely involving a combination of treatments to tackle spore heterogeneity most efficiently.

Germination heterogeneity and outgrowth efficiency

Recent advances in single cell techniques have allowed for heterogeneity assessment of spore germination and outgrowth in a range of conditions, including exposure to (sub) lethal heat treatments and food-relevant concentrations of sorbic acid, a widely used food preservative [48[•],49[•]]. Flow cytometry (FCM) and cell sorting in 96-well microtiter plates was applied to assess lag times of single spores of *B. subtilis* [48[•]] subjected to relatively mild heat treatments. A clear correlation between the loss of DPA and the severity of the heat treatment was observed, while outgrowth was always affected by sub-lethal heat. The results further showed that variation in germination capacity was reduced after mild heat treatment, but increased upon severe heat treatment, although the molecular cause for this is yet unknown. Interestingly, heat treatment studies on *Clostridium botulinum* spores revealed that the history of spore treatment and the conditions at the time of germination and outgrowth clearly affect germination and outgrowth lag times [50]. In this, great variation in lag times with no clear relationship between lag times of different stages of the germination process were observed, similarly to earlier studies concerning the effect of changing salt concentrations during sporulation [51]. Specifically, a lower growth temperature (in the range 8–22 °C) predominantly affected outgrowth and doubling times, whereas spore heat treatments at 80 °C extended germination times rather than later growth stages [50]. An important message from these studies is that environmental factors during sporulation and pretreatments of spores affect lag time duration and variability and that data derived from such studies can be used in risk assessment to improve the prediction of food poisoning risks [50].

In a similar line of research van Melis *et al.* [49[•]] studied the impact of sorbic acid on germination and outgrowth of *B. cereus* spores. Sorbic acid (SA) was found to have a profound affect on the germination of these spores. Addition of a range of increasing food-relevant concentrations of un-dissociated sorbic acid (HSA) caused effects ranging from reduced germination and outgrowth to a complete block of germination. Flow cytometric and transcriptome analyses confirmed the affected phenotypes related to different stages in germination. The main findings of the transcriptome analysis at different HSA concentrations were the differential expression of genes related to outgrowth, cell envelope alterations and multi-drug resistance. The latter two responses have been observed previously in vegetative cells of *B. subtilis* in response to SA [52], although understanding the significance of this finding in relation to SA resistance requires further investigation. Both general and stress-specific (HSA-stress associated) genes determine spore outgrowth capacity under stress conditions. In view of the range of food preservation stresses applied, future research may be

Figure 2



Graphical representation of underlying causes and influences of spore heterogeneity. Environmental conditions influence gene transcription in bacterial populations and affect intracellular levels of protein modification as well as the ratio of active and non-active regulator proteins, such as activators, repressors, and/or sigma factors. This can result in differences in gene expression (levels) in forespores of the same, clonal population of cells during the sporulation process. Slight differences in essential spore properties, such as spore coat composition, core water content, germinant receptor levels, or core lytic enzyme levels, give rise to a heterogeneous spore population (indicated by different patterned fillings). These spores may respond differently to administered treatments or germination triggers in the form of heat, pressure, acids, or nutrients, which leads to heterogeneous germination and/or outgrowth behavior. Recent developments in single cell analysis of spore populations have led to a tremendous enhancement in our understanding of the factors involved as well as of regulatory mechanisms and the influence of environmental factors. Eventually, this may increase our ability to predict the level of phenotypic heterogeneity in *Bacillus* spore populations and their responses to specific triggers or treatments and therefore aid in the improvement of predictive models for food preservation.

aimed at the identification and characterization of stress and damage repair factors and their impact on heterogeneity in outgrowth capacity at relevant conditions including (model) foods. This may lead to identification of markers for repair and survival, leading to new concepts for enhanced control of spore-forming bacteria.

Future directions

Cellular variability comprises an essential property for the survival of microbial populations and complicates effective strategies to control bacterial contamination of food and food processing facilities. The occurrence of so-called

superdormant spores [53••] with increased resistance to conventional preservation treatments and a requirement for heat treatment at higher temperatures [28•] adds to this complication, because alteration of conventional preservation treatments is limited by the requirement to maintain food quality.

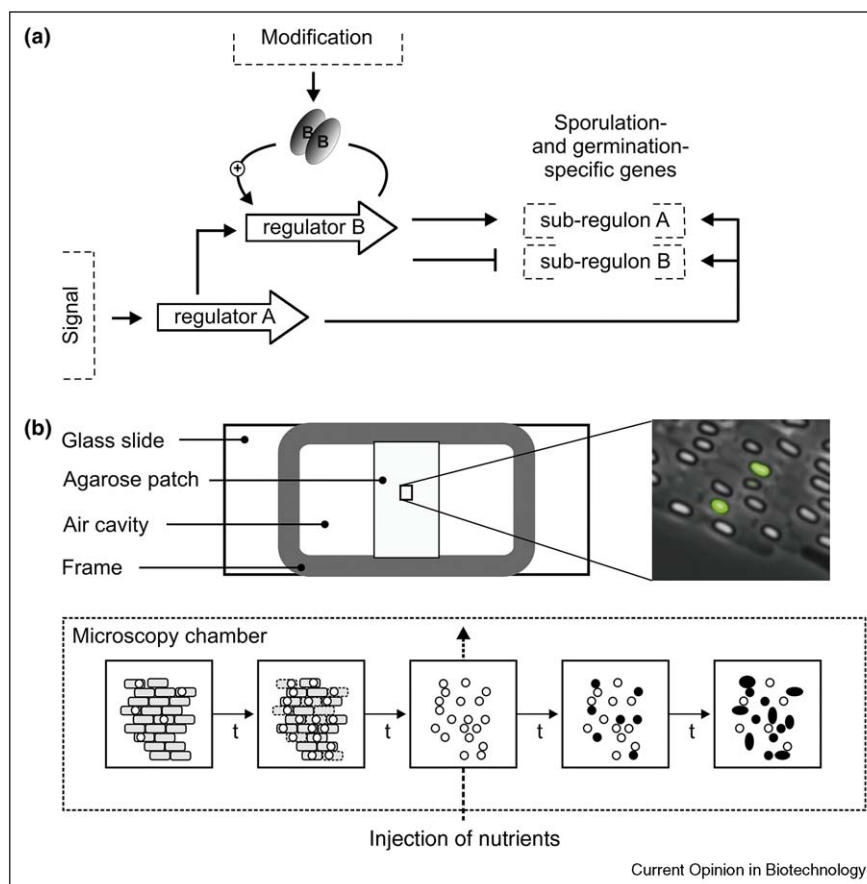
Observed differences in germination and outgrowth responses are without doubt the result of variations in specific spore properties, such as lower core water content [28•] and/or variable numbers of Ger receptor complexes [53••]. Therefore, an increased understanding of the

causes of heterogeneity in spore resistance mechanisms, germination and outgrowth will aid in the prediction of spore properties and behavior and may eventually lead to improved predictive models for food preservation. Furthermore, validating these data in relevant food models is an essential element in this process, as different compositions of food stuffs affect spore germination and outgrowth properties. Obtaining an in-depth molecular understanding at the single cell level combined with how this correlates to spore behavior at the population level forms the challenge of future research. Implementation of novel or improved state of the art techniques, such as flow cytometry [54] and/or time lapse (fluorescence) microscopy [55] with the combined use of microfluidic devices [56], will allow for the analysis of relevant sporulation and germination associated processes in time (Figure 3). This will reveal how the history of conditions during growth

affects these two processes and thus will provide leads to counteract or change them. The importance of sporulation history on the heterogeneous outcome of specific spore properties [16] as well as of spore germination and outgrowth [48*,49*,50,51] has been described extensively. Nevertheless, it must be stressed at this point that often the history of spores in food-related situations is not known, and even knowing the history does not automatically solve the problem. However, including different spore histories, while mapping germination efficiency and heterogeneity therein will provide a better representation of true environmental conditions rather than uniform laboratory conditions and is therefore in our opinion important to consider.

Combinations of above mentioned techniques with Raman spectroscopy and optical tweezers will allow for

Figure 3



Graphical representation of current approaches to study the molecular mechanism of germination heterogeneity in *Bacilli*. (a) Schematic representation of a random regulatory network that governs the expression of a sporulation and germination specific gene set. Stochastic fluctuations in gene expression and protein modification together with positive feedback regulatory mechanisms are indicated to represent the basis of phenotypic variation. Arrows indicate a positive, activating effect, whereas perpendiculars represent negative, repressing actions. Adapted from Smits *et al.* [7]. (b) Graphical representation of an experimental set up including time lapse fluorescence microscopy to study sporulation and germination heterogeneity on the single cell level. Cells are loaded on an agarose strip on a microscopy slide and allowed to grow and sporulate in a climate controlled chamber around the microscope. Cellular growth, sporulation and (heterogeneous) *gfp* expression (inset) under the control of a promoter of interest can be visualized in time. Theoretically, germinants could be added to the free-lying dormant spores using microfluidic devices (indicated by the dotted arrow), to initiate germination and eventually correlate germination efficiency to gene expression profiles while knowing the sporulation history of single cells.

the analysis of various molecular components and changes herein during different stages of spore germination [57]. Quantitative data obtained from such studies can furthermore be used to generate predictive models [45]. Eventually, combined efforts towards obtaining molecular, physiological, and quantitative data on spore variability and behavior in food models are required to improve control and hurdle strategies to ensure food safety and quality.

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